



FETUIN-A IS A BIOMARKER FOR DISEASE ACTIVITY AND TREATMENT EFFICACY IN MULTIPLE SCLEROSIS

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BACKGROUND

Fetuin-A (Alpha2-Hermans-Schmid glycoprotein) was identified in a cerebrospinal fluid (CSF) proteomics study that revealed a significant elevation of this protein in patients with multiple sclerosis (MS) compared to controls.

OBJECTIVES

We investigated whether fetuin-A levels in the CSF and brain correlated with disease activity in MS. We also examined if this protein could be used as a biomarker for treatment response to natalizumab

METHODS

Subjects for fetuin-A Analysis

CSF (n=100) and plasma (n=36) samples were obtained from patients at our center through an IRB-approved protocol. Active disease in MS was defined by the presence of any one of the following criteria in the 6 months preceding CSF sample collection: (1) one or more relapses; (2) change in 0.5 point or greater in EDSS (Expanded Disability Status Scale) score; and (3) change in MRI, specifically a change in the number or size of lesions or the presence of gadolinium enhancing lesions. Patients were excluded from analysis if there were any concurrent systemic illness and any significant alterations of their plasma protein levels. Patients receiving steroid therapy in the 3 months preceding sample collection were also excluded from analysis.

Sample collection for Natalizumab treated subjects

Patients receiving natalizumab were prospectively included in the fetuin-A analysis (n=78). At our center in addition to the clinical and radiological monitoring as mandated by the TOUCH[®] Prescribing program, we obtained plasma and CSF samples for screening for JC virus prior to patients commencing natalizumab and after 6 and 12 months of treatment with an IRB approved protocol. For data analysis, fetuin-A levels were correlated with treatment responses.

CSF and plasma collection

CSF samples were collected by lumbar puncture using standard sterile procedures. CSF was spun immediately after collection and cell free aliquots frozen at -70°C until analysis. Plasma samples were similarly stored.

Fetuin-A protein ELISA Analysis

Levels of fetuin-A in the CSF and plasma were determined using the human Fetuin-A ELISA kit according to the manufacturer's instructions (Biovendor). For quantification, serum samples were diluted 1:10,000 and CSF diluted 1:100. All samples were analyzed in duplicate and values extrapolated from a standard curve for each assay. The analytical limit of detection of Fetuin-A is 0.35ng/ml.

MS and Normal Brain Tissue

Fetuin-A protein expression was examined in 23 MS brain samples in plaques, and from areas of normal appearing white and grey matter. 10 control brains were similarly examined. 21 MS and 10 brain tissue specimen acquired from the Human Brain and Spinal Fluid Resource Center, Department of Veterans Affairs (Los Angeles, CA), and 2 samples were obtained from the Human Brain Bank of the Multiple Sclerosis Research Center of New York.

Immunohistochemistry

Immunohistochemical staining was performed using the avidin/biotin method on frozen and paraffin-embedded tissue sections. Briefly, after blocking sections were incubated with polyclonal anti-human (R&D Systems and Biovendro Laboratories) or anti-mouse Fetuin-A antibody (Santa Cruz Biotechnology Inc.). A biotinylated secondary antibody coupled with streptavidin-horseradish peroxidase was then used with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as a substrate. For myelin basic protein (MBP, Chemicon International) staining, appropriate anti-human and anti-mouse antibodies were used. Positive and negative controls were included with each experiment

Quantitative PCR for human fetuin-A mRNA in brain tissue

RNA was extracted from 10 µm frozen sections from 10 distinct plaques from 8 different MS brains. As control, RNA derived from the brain of 10 healthy donors was used. RNA was reverse transcribed into cDNA with random hexamers using the superscript RT kit (Invitrogen). The forward primer specific for the human Alpha2 HS-glycoprotein gene is: 5'-CTCAGCGGAGGACGTGGCAAGG-3' and the reverse primer is: 5'-TGAGCCCGGAGAAATTCCTCC-3'. The SYBR Green 1 kit from Roche was used. The cycle conditions were 95°C:10 mins followed 95°C:10 sec, 65°C:10 sec for 40 cycles. The expression of fetuin-A was compared to house keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) giving a normalized ratio.

T-cell proliferation assay

The effect of fetuin-A on T-cell proliferation was investigated *in vitro*, to determine if elevated fetuin-A levels correlated with TGFβ inhibition. Peripheral blood mononuclear cells were isolated from 10 subjects by standard procedure using a Ficoll-Paque density gradient. CD4+ T-cell were then purified by positive selection using CD4 microbeads (Miltenyi Biotec). The T-cells were cultured in serum-free AIM-V medium for 16 hours and cells stimulated with artificial APCs (aAPC) comprised of magnetic beads coated anti-CD3 and anti-CD28 (Invitrogen) at a ratio of 1:20 (aAPC:T-cell). TGFβ1 (1ng/ml) and fetuin-A (2ng/ml) was added to the T-cell cultures, BSA (2ng/ml) was used as a control protein. Before stimulation with aAPCs. CD4+ T-cell were labeled with CFSE (Invitrogen). After 3 days of stimulation, T-cell proliferation was assessed by flow cytometry.

RESULTS

CSF levels of fetuin-A in patients with active disease (mean=1600ng/ml) was significantly elevated (p=0.0003) in comparison to patients with stable disease (mean=1177ng/ml), while no differences were found in plasma fetuin-A levels (data previously presented). In brain tissue fetuin-A levels are markedly elevated in all areas of demyelination in comparison to other regions in the same brain and also by comparison to control brains. Fetuin-A mRNA expression levels were significantly higher in MS brain tissue compared to levels of normal brain (~40,000 fold increase; p<0.0001). Fetuin-A treatment resulted in a significant decrease in T-cell proliferation *in vitro* (p=0.0007). In natalizumab treatment responders CSF fetuin-A levels were significantly lowered post-treatment. In EAE, fetuin-A was specifically increased in acute inflammatory lesions.

CSF Fetuin-A levels Analysis

The characteristics of the 100 MS patients and mean fetuin-A levels of active and stable disease patients are shown in Table 1 and Figure 1.

Table 1. Patient Demographics

Determinants	Active Disease	Stable Disease
Number	50	50
F/M (ratio)	1.63:1	2.85:1
Mean Age	47	50
Age Range	28-70	26-68
Disease Duration (years)	1-39	5-32
Mean CSF Fetuin-A levels (ng/ml)	1599+/-628	1177+/-468
+/- standard deviation	P=0.0003	

Natalizumab treatment lower CSF fetuin-a levels

CSF fetuin-A levels of 78 patients were measured before treatment with natalizumab (baseline) and after 6 and 12 months of treatment. The mean concentration of fetuin-A was significantly reduced from 1602ng/ml to 1404ng/ml after 6 months (p=0.0013) to 1198ng/ml after 12 months (p<0.001) was shown in Figure 2.

CSF Fetuin-A Levels Pre- & Post-Treatment of Natalizumab

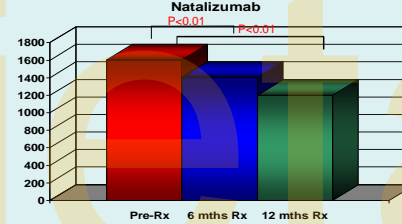


Figure 2 CSF fetuin-A levels before treatment with natalizumab and after 6 and 12 months treatment

Human MS Brain Fetuin-A Immunostaining

By immunohistochemistry, fetuin-A expression was increased in all MS plaques examined. The areas of increased fetuin-A coincided with the areas of demyelination by MBP immunostaining (Figure 3B). By contrast, fetuin-A in normal appearing white and grey matter of MS brains as well as in control brains, was not increased above background levels.

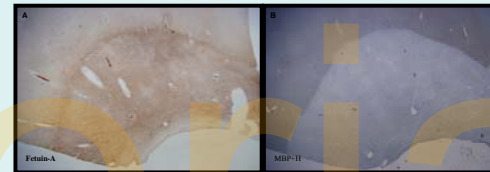


Figure 3 Increased fetuin-A immunostaining in areas of demyelination in MS brain as viewed by 2x light microscopy.

Fetuin-A mRNA levels are increased in MS brain tissue

To determine if the increased Fetuin-A protein levels in MS could be explained on basis of specific CNS expression, we assessed mRNA levels in MS and control brains. Fetuin-A mRNA expression levels were significantly higher in MS brain tissue in comparison to levels in normal brain (~40,000 fold increase; p<0.0001) as shown in Figure 4.



Figure 4 Fetuin-A mRNA levels in MS brain tissue and normal brain tissue

The anti-inflammatory effects on T-cell proliferation of TGF beta in vitro are partially antagonized by fetuin-A

TGFβ1 is a known inhibitor of CD4+ T-cell proliferation. We investigated whether the addition of fetuin-A would affect this inhibition. By FACS analysis for a representative sample in Figure 5, TGFβ1 alone resulted in a 23.5% reduction in T-cell proliferation, whereas in the presence of fetuin-A only 16.4% reduction was seen. There is a significant antagonism of TGFβ1 by fetuin-A (p=0.0007) shown in Figure 5. As a control, BSA substituted for fetuin-A had no discernible effect on TGFβ1 activity (data not shown).

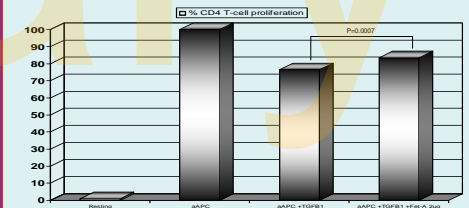


Figure 5 Fetuin-A induces a significant increase in T-cell proliferation in the presence of TGFβ

CONCLUSIONS

- In natalizumab-treated patients CSF fetuin-A levels were significantly lowered post-treatment.
- In brain tissue fetuin-A levels are markedly elevated in all areas of demyelination in comparison to other regions in the same brain and also by comparison to control brains.
- Significantly higher expression of fetuin-A mRNA occurred in MS brains compared to control.
- In EAE fetuin-A was specifically increased in acute inflammatory lesions.
- Fetuin-A causes a significant increase in T-cell proliferation in the presence of TGFβ1. Fetuin-A is an antagonist of TGF-β and thus may promote inflammatory activity
- These findings suggest that fetuin-A is a biomarker of disease activity in MS and have application as an indicator of therapeutic efficacy.

This study was supported by the Advisory Board of the MSRCNY. The authors have no disclosures